Leukemia (2017) **31,** 2824–2832

www.nature.com/leu

OPEN

ORIGINAL ARTICLE

RNA editing signature during myeloid leukemia cell differentiation

C Rossetti[1], E Picardi[2,3], M Ye[1], G Camilli[1], AM D'Erchia[2,3], L Cucina[4], F Locatelli[4,5], L Fianchi[6], L Teofili[6], G Pesole[2,3], A Gallo[4] and R Sorrentino[1]

Adenosine deaminases acting on RNA (ADARs) are key proteins for hematopoietic stem cell self-renewal and for survival of differentiating progenitor cells. However, their specific role in myeloid cell maturation has been poorly investigated. Here we show that ADAR1 is present at basal level in the primary myeloid leukemia cells obtained from patients at diagnosis as well as in myeloid U-937 and THP1 cell lines and its expression correlates with the editing levels. Upon phorbol-myristate acetate or Vitamin D3/granulocyte macrophage colony-stimulating factor (GM-CSF)-driven differentiation, both ADAR1 and ADAR2 enzymes are upregulated, with a concomitant global increase of A-to-I RNA editing. ADAR1 silencing caused an editing decrease at specific ADAR1 target genes, without, however, interfering with cell differentiation or with ADAR2 activity. Remarkably, ADAR2 is absent in the undifferentiated cell stage, due to its elimination through the ubiquitin–proteasome pathway, being strongly upregulated at the end of the differentiation process. Of note, peripheral blood monocytes display editing events at the selected targets similar to those found in differentiated cell lines. Taken together, the data indicate that ADAR enzymes play important and distinct roles in myeloid cells.

Leukemia (2017) **31,** 2824–2832; doi:10.1038/leu.2017.134

INTRODUCTION

RNA editing is an important posttranscriptional process able to increase transcriptome and proteome.[1–3] In humans, the most common type of RNA editing is mediated by ADAR enzymes, which convert adenosine into inosine within double-stranded RNA (dsRNA). This modification is mediated by two adenosine deaminases acting on dsRNA: ADAR1 (ADAR) and ADAR2 (ADARB1) whose function is tightly regulated. ADAR1 has at least two protein isoforms, a constitutive p110 and an inducible p150. Although p110 is localized in the nucleus, the p150 isoform, thanks to its nuclear export sequence, is also present within the cytoplasm.[4,5] A-to-I editing is pervasive in *Alu* elements due to their ability to form dsRNA structures.[6] How dsRNA structures are formed and whether there are 'flag' sequences that allow ADAR enzymes to identify the possible targets is matter of intense studies.[7,8] As inosine is read as guanosine by splicing and translation machineries, ADARs can also alter splicing patterns and change amino-acid sequence. Genomic ablation of either Adar1 or Adar2 in mice is lethal, indicating that both these enzymes are essential *in vivo*.[9] Several pathological conditions were also linked to ADAR dysfunctions, such as neurological disorders, autoimmunity, cancer and viral infections.[10–16]

In particular, ADAR1 has been shown to be essential for proliferation and differentiation of cells of both erythroid and myeloid lineage.[17,18] Lineage commitment toward myeloid differentiation is a complex, multistep mechanism marked by distinct transcriptional and translational changes, including the expression of specific cell surface markers and epigenetic modifications leading to typical morphological changes.[19] Here we investigated the modulation of ADARs expression and activity in myeloid leukemia cells throughout differentiation along the monocyte/macrophage axis, with focus on recoding sites in differentiated cells.

MATERIALS AND METHODS

Cells

Human histiocytic leukemia U-937 (ATCC CRL-1593.2, Manassas, VA, USA) and human acute monocytic leukemia THP1 (ATCC TIB-202) cell lines were treated for the indicated time with GM-CSF (25 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) plus 1,25-dihydroxyvitamin D3 (vitD3 10 nM; Sigma-Aldrich, Oakville, ON, Canada) or with phorbol-myristate acetate (PMA) (80 nM for U-937 and 100 nM for THP1; Sigma-Aldrich). In some experiments, cells were treated with 10 μM of MG132 (Calbiochem, San Diego, CA, USA) for 6–12 h. HeLa cells (ATCC CCL-2) were treated for 96 h with PMA (80 nM). Monocytes purified from peripheral blood mononuclear cells of seven healthy volunteers (PBMC) were selected with anti-CD14 mAb coupled to magnetic beads. Collected cells were incubated with saturating concentration of allophycocyanin (APC)-conjugated CD14, CD11B (Miltenyi Biotec), CD54 (Immunotools GmbH, Friesoythe, Germany) at 4 °C for 30′ and suspended in PBS/1% paraformaldehyde. Fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson, Missisauga, ON, Canada) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Blast cells from 13 patients with acute myeloid leukemia (AML) admitted at the Hematology Department of the 'A. Gemelli' Hospital (8 males and 5 females, mean age 68 years, range 40–84) were isolated from either bone marrow or peripheral blood by density gradient centrifugation (lymphocyte; Cedarlane Laboratories, Burlington, ON, Canada). The purity of leukemic cells was always higher than 98%. In one case, cryopreserved cells were also available and used for differentiation experiments. The study was approved by the local Institutional Review Board.

[1]Department of Biology and Biotechnology 'Charles Darwin', University La Sapienza, Rome, Italy; [2]Department of Biosciences, Biotechnology and Biopharmaceutics, University of Bari, Bari, Italy; [3]Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies of the National Research Council, Bari, Italy; [4]Department of Pediatric Oncohaematology, Bambino Gesù Children's Hospital IRCCS, Rome, Italy; [5]Department of Pediatric Science, University of Pavia, Pavia, Italy and [6]Institute of Hematology, Catholic University, Rome, Italy. Correspondence: Professor R Sorrentino, Department of Biology and Biotechnology 'Charles Darwin', University La Sapienza, Via die Sardi 70, Rome, Italy.
E-mail: rosa.sorrentino@uniroma1.it

Received 18 November 2016; revised 23 March 2017; accepted 19 April 2017; accepted article preview online 9 May 2017; advance online publication, 2 June 2017

RNA isolation and real-time-PCR analysis

Total RNA was isolated by TRIzol (Invitrogen, Beverly, MA, USA; Thermofisher Scientific, Waltham, MA, USA), DNase-treated (Invitrogen) and quantified with an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). cDNAs were generated by SuperScript II reverse transcriptase (Invitrogen) using random hexamers or specific primers (Supplementary Table S1). Gene-specific exon–exon boundary PCR products (TaqMan gene expression assays, Applied Biosystems, Thermofisher Scientific, MA, USA) were measured with a PE Applied Biosystems ABI PRISM 7300 using TaqMan 2X Universal Master Mix and the TaqMan Gene Expression Assays. Cytokines were quantified using specific primers (Supplementary Table S1). Relative quantification was performed in duplicates from two independent real times according to the 2-ΔΔCt method and normalized on GAPDH. The primers were supplied by Applied Biosystems: GAPDH, ID Hs99999905; ADAR2, ID Hs00953730_m1 and ADAR, ID Hs00241666_m1 and were expressed in arbitrary units.

Analysis of RNA editing

A-tol/G RNA editing was detected by direct sequencing of RT-PCR products. The editing event is given by a mixture of A+G peaks in the sequence chromatogram, and measured calculating the ratio between the area of the peak corresponding to G and the sum of the areas of the double peaks A+G. Sequence analysis was performed with the software Bioedit and, for each sample, two/three independent RT-PCR were carried out.

Cell transfection

HEK 293T cells (7×10^5), showing an undetectable endogenous editing activity, were transiently transfected using lipofectamine 2000 (Invitrogen), with either 4 μg of EGFP-ADAR1 or EGFP-ADAR2. Human astrocytoma cell line U118 (ATCC HTB-15), stably transfected with EGFP vector or EGFP-ADAR2 as already reported,[20] was also used. The stably ADAR1-silenced U-937 cell line was obtained using the BLOCK-iTTM inducible Pol II miR RNAi Expression Vector Kit by EmGFP (Invitrogen).

Western blot analysis

The following primary antibodies were used for immunoblotting: anti-ADAR1 (Bethyl Laboratories, Inc, Montgomery, TX, USA), anti–ADAR2 (Sigma-Aldrich), anti-p21 and anti-GFP (Santa Cruz Biotecnology, Santa Cruz, CA, USA), anti-β-catenin (Cell Signaling Technology, Beverly, MA, USA). Secondary antibodies for chemiluminescence were: anti-mouse or anti-rabbit IgG, HRP-linked Abs (GE Healthcare Life Sciences, Salt Lake city, UT, USA). Western blot assays were analyzed using the Pierce ECL system (Thermo Scientific). Protein levels were normalized for GAPDH using ImageJ version 1.45 software.

Strand-oriented RNA sequencing

Cytoplasmic rRNA removal was performed for each total RNA sample using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). The stranded-oriented RNA-seq library was prepared using the TruSeq Stranded Total RNA Sample Prep Kit (Illumina, San Diego, CA, USA). Briefly, each RNA was chemically fragmented before the random priming reverse transcription reaction for first strand cDNA generation. The fragmentation step resulted in a RNA-seq library including inserts ranging in size ~100–400 bp. During the second strand synthesis, deoxyuridine triphosphate (dUTP) was incorporated in place of deoxythymidine triphosphate (dTTP), thus preventing amplification of this strand during the subsequent PCR step and retaining strand information. cDNA libraries were sequenced on the Illumina Next Seq 500 platform. Paired-end reads of 100 nt were generated for each fragment.

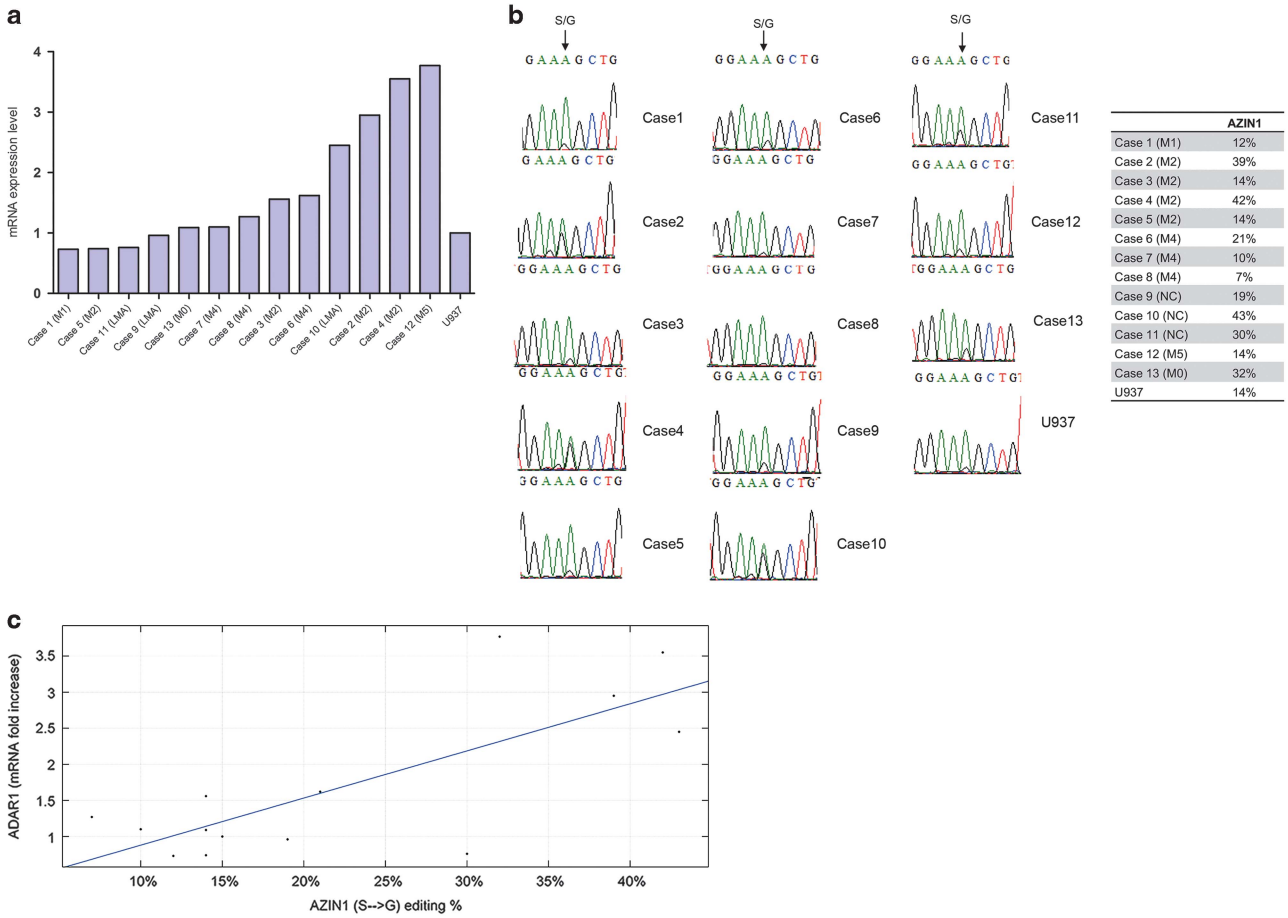

Figure 1. Correlation of editing and ADAR1 RNA expression in primary AML blasts. (**a**) Variation in the expression of ADAR1 mRNA in blasts from 13 AML cases represented as fold increase compared to U-937 cells. (**b**) Percentage of the editing observed in AZIN1 transcripts (**c**) Correlation index between ADAR1 mRNA expression and the editing of AZIN1 ($r = 0.775$). The French-American-British classification (FAB) subtype for each case is reported. NC, not classified.

RNA-seq alignment and gene expression analysis

RNA-seq reads in FASTQ format were inspected using FASTQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low-quality regions (Phred cutoff of 20) were trimmed using TrimGalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), excluding reads with final length < 50 bases. Cleaned reads were subsequently aligned onto the complete GRCh37 human genome by means of GSNAP,[21] providing a list of exon–exon junctions from Ensembl, UCSC and RefSeq databases. Unique and concordant alignments in SAM format were converted in the binary BAM format by means of SAMtools.[22] Transcriptome quantification and differential expression was performed using Cufflinks[23] and CuffDiff2[ref. 24] software through the RAP web-service (https://bioinformatics.cineca.it/rap/).[25]

To identify sets of genes that are both strongly correlated to expression data and functionally related to their gene ontology (GO) annotations, we used the GO-PCA (principal component analysis) method (PMID: 26575370). In brief, it adopts a two-step approach in which PCA is performed first. Then, each principal component is tested for whether it is driven by functionally related genes (in the form of gene ontology annotations).

RNA editing candidate gene analysis

RNA editing candidates were detected using REDItools.[26] The Alu editing index (AEI), the weighted average editing level across all expressed Alu sequences, was calculated using custom scripts according to the methodology described in Bazak *et al.*[27] RNA editing in recoding sites was assessed using REDItools and providing a list of 2955 known positions from REDIportal database[28] in which RNA editing causes amino-acid change. This initial list was filtered in order to include only RNA editing positions supported by RNA-seq reads in all replicates. In addition, we required at least a replicate per sample with a minimum coverage of 10 reads. For each sample, we calculated the average editing level and selected only positions in which the RNA editing difference between sample at 0 h and sample at 96 h was higher than 4%. The selection of recoding editing sites was performed using custom scripts.

Statistical analysis

Results are expressed as mean and standard error (± s.e.m.) from at least three independent experiments. Statistical significance (*P*-values) of differences between mean or median values was evaluated using the Student's *t*-test, the Mann–Whitney *U*-test or Kruskal–Wallis one-way analysis, whenever indicated, using GraphPad Prism.

Data availability

RNA-seq data have been submitted to SRA database under the accession SRP103305 (BioProject: PRJNA381911).

RESULTS

Analysis of ADAR expression and activity in primary myeloid leukemia cells

It is generally acknowledged that ADAR1 plays an important role in the maintenance of self renewing progenitor leukemia cells. However, very little is known about the activity of ADAR enzymes in primary AML blasts. Here we analyzed the mRNA expression and activity of ADAR1 in AML blasts obtained from 13 patients. Of

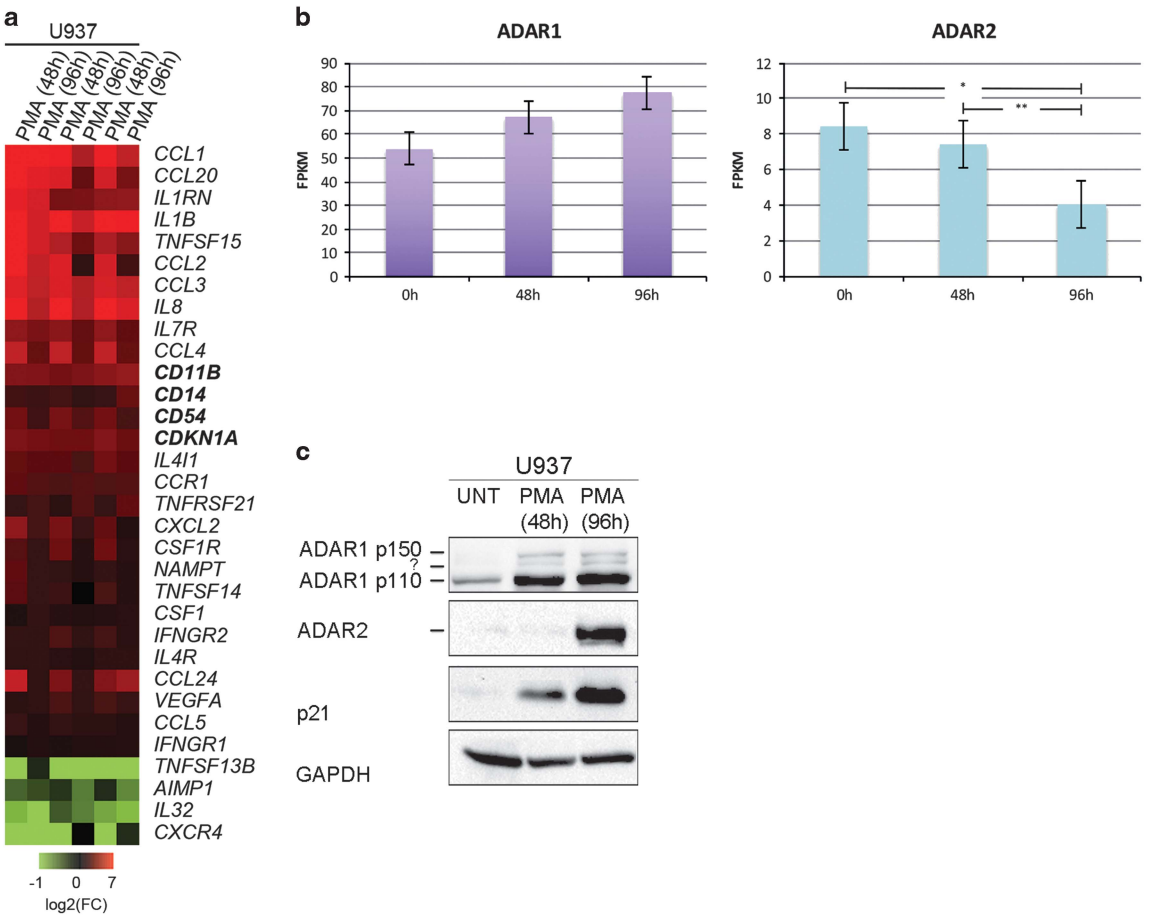

Figure 2. PMA treatment in U-937 cells induces the expression of both ADAR1 and ADAR2. Total RNA extracted from triplicates of U-937 cells, treated with PMA (80 nM) for 48 or 96 h, underwent RNA-seq analysis. (**a**) RNA expression level of selected genes belonging to the inflammatory/differentiation pathways (triplicates). (**b**) ADAR1 and ADAR2 expression (FPKM, triplicates). (**c**) One representative of three western blots of ADAR1, ADAR2 and p21 in PMA-treated U-937 cells. $*P < 0.01$; $**P < 0.001$. FPKM, fragments per kilobase of exon per million fragments mapped.

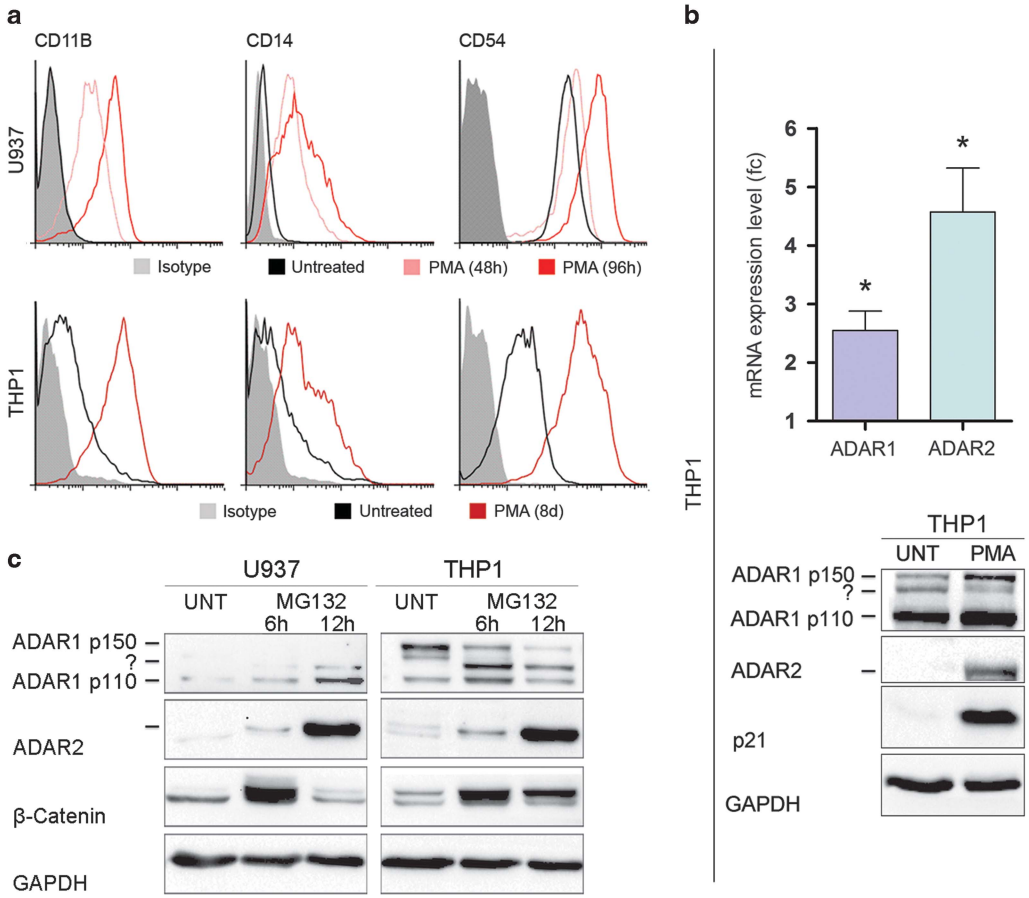

Figure 3. The expression of ADAR2 in both U-937 and THP1 cells is induced by PMA and downmodulated through the ubiquitin–proteasome pathway. (**a**) Analysis of the expression of CD11B, CD14 and CD54 monocyte/macrophage differentiation markers as detected by specific antibodies in undifferentiated and PMA-treated U-937 and THP-1 cells. (**b**) ADAR1 and ADAR2 mRNA variation from three independent experiments. mRNA is expressed as $\log_2$-fold increase. Below: one representative of three western blots of ADAR1, ADAR2 and p21 in THP1 cells before and after 8 days PMA treatment. (**c**) One representative of two western blots of ADAR1, ADAR2 and β – catenin[49] in U-937 and THP1 cells treated with MG132 (10 μg/ml) for the indicated times. $^*P < 0.05$.

note, ADAR1 mRNA was found to be expressed (from 0.73- to 3.77-fold compared to U-937 cells) in primary blasts, regardless of AML subtype (Figure 1a). In contrast, ADAR2 mRNA was barely detectable in blasts (data not shown). We then analyzed the editing levels at one well-known target of ADAR1, AZIN1,[29] in the 13 blast cells as well as in U-937 myelomonocytic cell line and found a variable percentage (Figure 1b) of the editing at adenosine recoding the residue 367 (serine to glycine) of AZIN1. Of note, editing at this site strongly correlated ($r = 0.775$) with ADAR1 mRNA expression (Figure 1c).

ADARs expression during myeloid cell differentiation

We therefore asked whether the expression and the activity of the ADARs were modified during differentiation. Triplicates of U-937 cells were differentiated by PMA[30–32] and totRNA extracted at 0, 48 and 96 h post induction for molecular analysis and deep-sequencing profiles. A time course of the U-937 cell morphology changes and ADAR1 expression is shown in Supplementary Figure S1. Time points at 0, 48 (time at which ADAR1 is detectable) and 96 h (time at which ADAR1 expression is the highest) have been chosen to perform RNA-seq analysis.

RNA signature of the cells (Supplementary Figure S2A) displayed upregulation of inflammatory pathway on cell differentiation (CCL1, CCL20, CCL2, CCL3, IL8 and CCL4) (Figure 2a), which has been also confirmed by qPCR (Supplementary Figure S3), CDKN1A (p21), a well-known marker for myeloid cell maturation,[33] was also upregulated as confirmed by protein expression (Figure 2a). GO-PCA analysis (PMID: 26575370) based on an unsupervised method to explore gene expression data using prior knowledge in the form of GO annotations, revealed 46 specific signatures, consisting of small set of genes that were strongly correlated by their expression as well as functionally related by GO annotations. All 46 signatures ($P < 0.001$) contained between 5 and 23 genes and were mainly related to inflammation and cell cycle (Supplementary Figure S2B and Supplementary Table S2).

Interestingly, we observed a trend of progressive increase of ADAR1 mRNA during differentiation, whereas ADAR2 mRNA was expressed ~10 times less compared to ADAR1 with a further decrease during differentiation (Figure 2b). Accordingly, the expression of the two ADAR1 isoforms (p150 and p110) were both increased during differentiation, whereas ADAR2 had an opposite trend undergoing a strong increase at 96 h post differentiation, time at which the cells acquired a macrophage-like phenotype (Supplementary Figure S1 and Figure 2c).

An additional myeloid cell line, THP1, was also PMA treated. After 8 days post treatment, these cells acquired a mature phenotype (Supplementary Figure S1) expressing CD11B, CD14 and CD54 at a level comparable to that observed in U-937 cells (Figure 3a). However, differently from U-937, THP1 increased ADAR2 mRNA during differentiation, whereas ADAR1 and ADAR2 proteins showed an expression profile similar to that observed in the U-937 (Figure 3b).

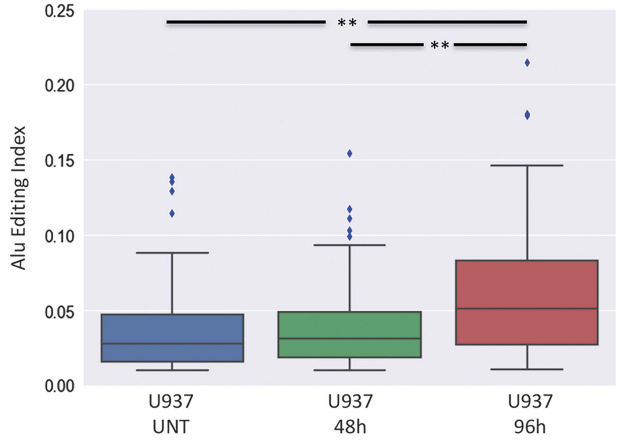

Figure 4. Analysis of the editing index in triplicates of U-937 cells untreated or PMA treated for 48 and 96 h. The AEI indicating the editing activity of ADARs was calculated in triplicates of U-937 cells untreated and PMA treated at 48 and 96 h, across all expressed Alu sequences. The statistical significance has been calculated by Mann–Whitney test correcting *P*-values with Benjamini–Hochberg procedure. Corrected *P*-values for each comparison are as follows: U937-UNT versus U937-48h, $P = 0.2627$; U937-UNT versus U937-96h, $P = 0.003$; U937-48h versus U937-96h, $P = 0.001$. $^{**}P < 0.01$.

To investigate why ADAR2 protein increases at the later stage of cell differentiation independently of its mRNA, we analyzed whether posttranslational mechanisms could play a role in these cells. Previous studies using mouse embryo fibroblasts had shown that ADAR2 can undergo proteasome degradation.[34] We therefore treated U-937 and THP1 cells with the proteasome inhibitor MG132 (Figure 3c). Interestingly, while ADAR1 level increased slightly in the U-937 cells and not at all in the THP1, ADAR2 progressively accumulated in both cell lines (Figure 3c).

Altogether, these results indicate that both ADAR enzymes are expressed during leukemia cell differentiation, with ADAR1 being present and active from the proliferative to the differentiated stage and with ADAR2 protein appearing throughout differentiation.

RNA editing signature during (PMA)-cell differentiation

Global A-to-I RNA editing was evaluated through the AEI as it represents the weighted average editing level across all expressed *Alu* sequences.[35] We observed that, in U-937 cells, the AEI value significantly increased ($P < 0.01$) from 48 to 96 h (Figure 4 and Supplementary Figure S4). The analysis of 171 A-to-I editing sites resulting in amino-acid substitutions, filtered from an initial pool of 2955 recoding positions from REDIportal database,[28] revealed variation in RNA editing levels along the differentiation process (Supplementary Table S3). Five recoding candidates, showing major variation in editing (AZIN1, CCNI, COG3, RHOA and SRP9), and two additional positions in the 3′UTR of RAB2B (selected for being over edited in the final step) and in the intronic region of PDE8A (known to be a target of ADAR2 in other cellular systems),[36,37] were experimentally verified (Table 1 and Supplementary Figure S7). In particular, the editing of AZIN1, edited by ADAR1 at amino-acid position 367,[29] increased during differentiation (Table 1) up to ~38% in U-937 and ~24% in THP1. Other genes, such as COG3, CCNI, RAB2B and RHOA, the last at four amino-acid positions, were also highly edited when cells were fully differentiated (Table 1). Of note, due to editing, RHOA displayed two consecutive recoding sites (Y175C and R176G) in exon 4, suggesting a major functional outcome.

Overall, these data, together with the analysis of the AEI, indicated that the editing activity of the ADARs increases during myeloid cell differentiation.

Table 1. RNA editing levels (%) in seven substrates on PMA or vitD3+GM-CSF treatment in U-937 and THP1 cells and monocytes

ADARs edited sites (% of editing ±s.e.m.)	*Chr*	*Position*	*aa position*	*A->A*	*U-937*		*U-937*			*THP-1*		
					Predicted		*Experimental*					*Monocytes*
					UNT	*PMA (96 h)*	*UNT*	*PMA (96 h)*	*VitD3+GM-CSF*	*UNT*	*PMA (8 days)*	*VitD3+GM-CSF*
AZIN1	8	103841636	367	S->G	6.7 (±0.3)	29.0 (±3.5)	13.7 (±1.8)	38.2 (±9.8)	33.2	5.2	23.7	31.2	35.4 (±6.3)
COG3	13	46090371	635	I->V	17.0 (±3.0)	37.1 (±3.0)	14.5 (±3.5)	26.5 (±8.5)	21.1	13.0	67.1	41.1	50.7 (±4.8)
CCNI	4	77979680	75	R->G	7.0 (±0.6)	39.3 (±1.8)	9.3 (±1.0)	39.3 (±9.8)	22.3	0	34.4	40.9	23.0 (±3.2)
SRP9	1	225974614	64	I->M	69.3 (±3.4)	73.7 (±1.2)	78.2 (±2.0)	77.7 (±1.2)	ND	54.1	77.2	ND	70.3 (±4.9)
		225974645	75	S->G	31.1 (±4.0)	23.0 (±3.8)	33.3 (±0.7)	36.7 (±0.6)	ND	23.2	26.0	ND	39.0 (±3.1)
RHOA	3	49398382	176	R->G	46.4 (±5.0)	33.0 (±12.7)	35.5 (±5.5)	47.0 (±5.0)	37.2	29.3	50.3	39.9	17.8 (±2.5)
		49398384	175	Y->C	30.1 (±4.2)	46.7 (±8.8)	22.9 (±2.1)	28.4 (±4.6)	27.4	16.6	48.9	42.1	18.7 (±1.9)
		49398394	171	S->G	29.7 (±10.1)	38.0 (±4.6)	34.8 (±1.2)	63.5 (±4.2)	32.1	11.9	45.0	40.3	18.3 (±4.1)
		49398423	162	K->R	24.2 (±14.5)	46.3 (±1.3)	1.8 (±1.6)	52.5 (±1.5)	22.2	22.1	50.1	30.8	27.5 (±5.5)
RAB2B	14	21928501	—	—	12.2 (±2.6)	52.5 (±5.5)	27.5 (±6.5)	41.3 (±3.0)	ND	21.4	51.2	ND	37.3 (±5.0)
PDE8A	15	85639918	—	—	ND	ND	12.6 (±2.8)	29.2 (±9.8)	ND	23.8	67.4	ND	82.2 (±4.1)

Abbreviations: ADAR1, adenosine deaminase acting on RNA1; Chr, chromosome; ND, not determined; PMA, phorbol-myristate acetate. UNT, untreated; Vitd3+GM-CSF, vitamin D3+granulocyte macrophage-colony stimulating factor.

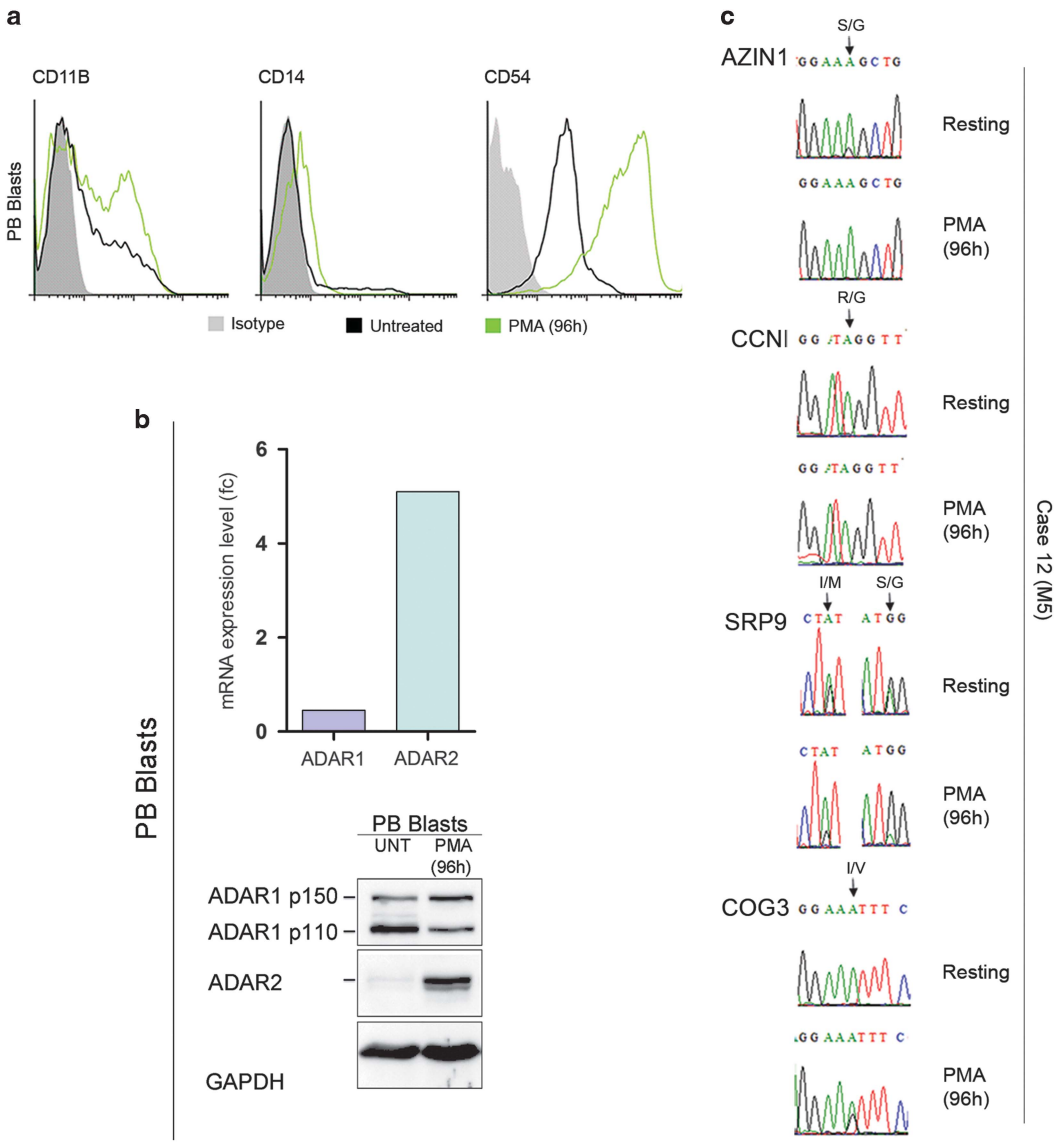

Figure 5. PMA treatment in AML cells induces the expression of ADAR2. AML blasts (M5) were exposed for 96 h to PMA. (**a**) Differentiation markers at baseline and after 96 h treatment. (**b**) RNA and protein expression of ADAR1 and ADAR2. mRNA is expressed as log_2-fold increase (**c**) Variation in the percentage of editing in four selected targets.

To verify that the increased activity of the ADARs was indeed a marker of differentiation, RNA editing was also analyzed both in cell lines differentiated by vitD3+GM-CSF and in peripheral blood monocytes, which represent the physiological final stage of myeloid cells maturation.

Treatment for 96 h of vitD3+GM-CSF-induced maturation of both U-937 and THP1, which acquired an intermediate phenotype between untreated and PMA-differentiated cells (Supplementary Figure S5). Specifically, CD14 was expressed at higher level indicating a differentiation toward monocyte rather than macrophage phenotype (Supplementary Figure S5A). Cells were still progressing through the cell cycle (Supplementary Figure S5B) and p21 was only moderately upregulated (Supplementary Figure S5C). The expression of both ADAR proteins was increased (Supplementary Figure S5C), concomitantly with the editing level of the selected substrates (Table 1 and Supplementary Figure S8). Finally, we investigated ADAR1 expression in AML cells of monoblastic subtype M5 during *in vitro* exposure to PMA (case 12 in Figure 1). Cells acquired a cell morphology reminiscent of that of differentiated U-937 cells (not shown), with a similar pattern of expression of cell surface CD11B, CD14 and CD54 (Figure 5a). In contrast to what was observed in U-937, in primary AML cells, PMA exposure did induce ADAR2 (both mRNA and protein) but not ADAR1 (Figure 5b). Consistently, editing at AZIN1 and CCNI sites, mainly edited by ADAR1, did not increase on PMA exposure, whereas SRP9 (aa position 64) and COG3 did, suggesting that they could be targeted by ADAR2 (see below) (Figure 5c).

To further confirm that what we have observed was specific for myeloid cell differentiation, we repeated the experiments using HeLa cells treated or not with PMA: As shown in Supplementary Figure S6, ADAR1 is not detectable in our conditions, whereas ADAR2 is present at time 0 and it does not increase significantly at 96 h. IL-1B is not produced at any time and p21 is not upmodulated. In accordance, the editing level of AZIN1 and CCNI is maintained low, whereas the editing at COG3 and SRP9 sites remained high.

Of note, monocytes showed RNA editing (as tested at specific sites) at a level comparable to that observed in the PMA-finally differentiated cell lines (Table 1).

Overall, these observations indicated that an increased activity of the ADARs characterizes mature myeloid cells.

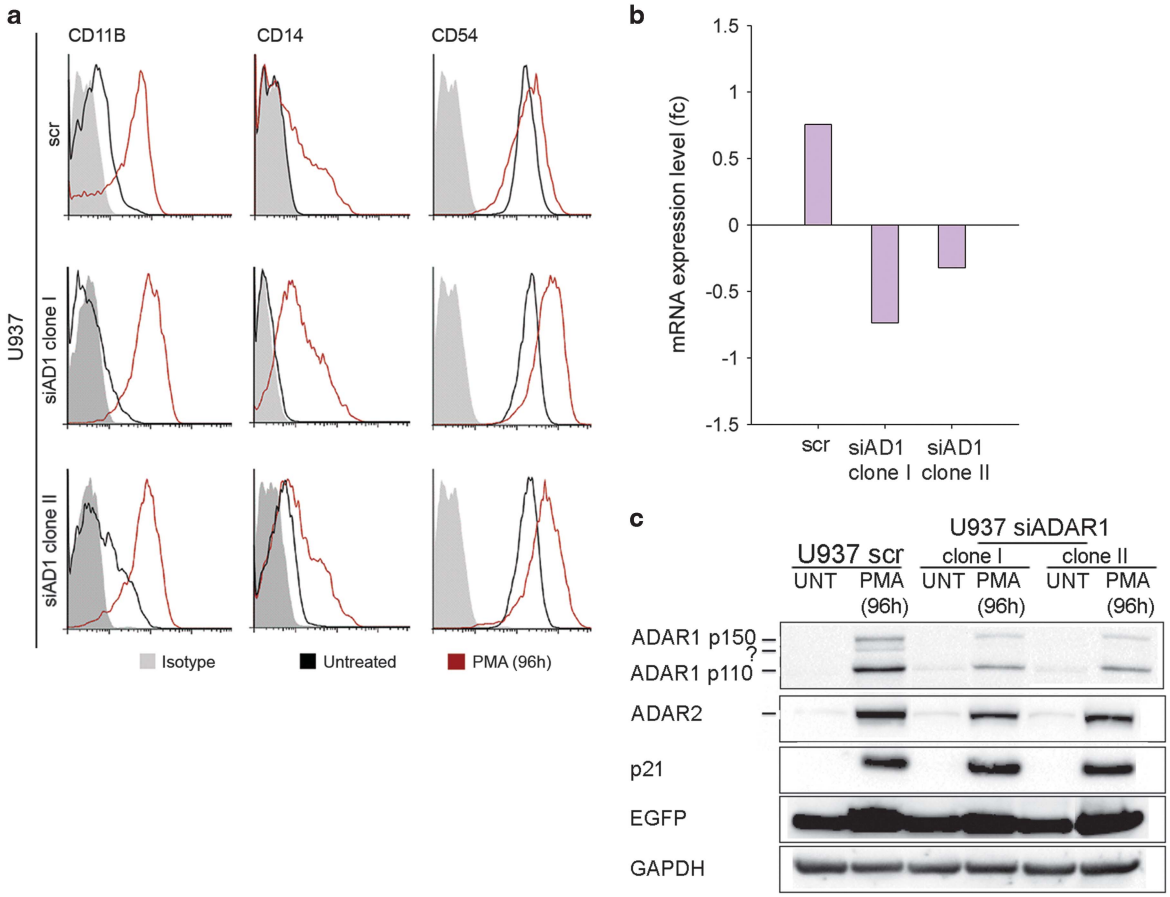

Figure 6. ADAR1 silencing in PMA-treated U-937 cells does not influence differentiation or ADAR2 activity. (**a**) Expression of the differentiation markers CD11B, CD14 and CD54 in the two si-ADAR1 clones undergoing PMA treatment. (**b**) Variation of ADAR1 mRNA expressed as $\log_2$-fold increase in the scramble (scr) and in the two transfected U-937 clones. (**c**) Variation in ADAR1, ADAR2 and GFP protein expression in the scramble and in the two si-ADAR1 clones.

Table 2. Modification in the level of RNA editing of seven substrates on PMA treatment in si-ADAR1 U-937 cells and controls

Edited transcript	*U-937 scr*		*U-937 si-ADAR1*				*HEK*			*U118*	
	UNT	*PMA (96 h)*	*UNT*		*PMA (96 h)*		*pEGFP*	*ADAR1*	*ADAR2*	*WT*	*ADAR2*
			1° clone	*2° clone*	*1° clone*	*2° clone*					
AZIN1	13%	30%	9%	10%	12%	15%	ND	ND	ND	ND	ND
CCNI	13%	27%	11%	13%	13%	12%	3%	60%	22%	11%	10%
RAB2B	21%	55%	27%	ND	11%	ND	10%	35%	25%	11%	11%
SRP9 (225974614)	79%	82%	82%	ND	81%	ND	52%	51%	68%	44%	73%
SRP9 (225974645)	21%	26%	33%	ND	24%	ND	13%	11%	14%	14%	18%
COG3	16%	57%	27%	24%	44%	40%	13%	17%	49%	21%	75%
PDE8A1	39%	66%	24%	29%	37%	50%	ND	ND	ND	19%	70%
RHOA (49398382)	16%	64%	14%	ND	25%	ND	16%	15%	18%	40%	58%
RHOA (49398384)	24%	50%	0%	ND	35%	ND	16%	16%	16%	40%	53%
RHOA (49398394)	39%	46%	0%	ND	36%	ND	14%	14%	23%	29%	88%
RHOA (49398423)	15%	40%	0%	ND	22%	ND	13%	19%	22%	17%	44%

Abbreviations: ADAR1, adenosine deaminase acting on RNA1; HEK, human embryonic kidney cells 293T; ND, not determined; pEGFp, plasmid enhanced green fluorescent protein; PMA, phorbol-myristate acetate. UNT, untreated.

Silencing of ADAR1 during differentiation abrogates the editing of specific substrates

We wanted to further dissect the activity of the two ADARs in our system. Considering that ADAR1 expression anticipates that of ADAR2, we attempted to stably silence ADAR1 in U-937 cells, using silencing constructs that carry both ADAR1–shRNA and eGFP under the cytomegalovirus (CMV) promoter, as it has been reported that PMA is able to induce specifically this promoter.[38] The use of PMA has therefore the advantage to block simultaneously the cell cycle, ADAR1 and cell death through NF-

KB and one of its targets, BCL2A1,[39] a member of the BCL-2 family whose expression has been found highly increased in the RNA-seq analysis of the PMA-treated U-937 (not shown). We observed that ADAR1 silencing led to massive cell death in our system. However, after many attempts, we finally managed to isolate two U-937 clones (si-clones) stably expressing the ADAR1–shRNA GFP, but still producing a detectable amount of ADAR1. The phenotype of the PMA-differentiated si-ADAR1 clones was similar to the one observed in U-937 controls, as verified by CD11B, CD14 and CD54 expression (Figure 6a). On differentiation, p21, ADAR2 and eGFP were induced. As expected, in PMA-treated scr-U-937 cells, ADAR1 increased at both RNA (Figure 6b) and protein level (Figure 6c), while it appeared less expressed in the two PMA-treated silenced clones, due to the PMA-mediated boost of the ADAR1–shRNA. Compared with the control (U-937 scr), on PMA treatment the two si-clones did not increase the editing at some genes (AZIN1, CCNI and RAB2B) (Supplementary Figure S9 and Table 2), indicating that these were specific targets of ADAR1. To verify the contribution of ADAR2, we used HEK cells overexpressing either ADAR1 or ADAR2 and U118 cells overexpressing ADAR2.[40] This allowed to infer that the remaining genes were partially or exclusively targeted by ADAR2, that is, COG3.

We concluded that during monocyte/macrophage differentiation, both ADARs increase their activity and can independently contribute to the editosoma.

DISCUSSION

It is not generally clear whether ADAR1 and ADAR2 play the same or different roles in human cells. Our study focused on the role of ADAR enzymes in human myeloid leukemia cells, finding that they are both overexpressed in cells undergoing differentiation to monocyte/macrophage lineage. We found, however, that the two ADARs are differently regulated during myeloid differentiation: while ADAR1 is progressively modulated during maturation, ADAR2 undergoes a sharp increase during differentiation.

Of note, ADAR1 has been found to be required for normal hematopoiesis and for promoting malignant progenitor reprogramming in chronic myeloid leukemia.[41–44] Consistently, we observed that myeloid leukemia primary blasts from 13 AML patients, as well as two myeloid leukemia cell lines, express a detectable, although variable, amount of ADAR1. Moreover, we noticed that U-937 cells, stably expressing an ADAR1-silencing vector, were also retaining a small amount of ADAR1, indicating that some activity of ADAR1 is necessary for blasts maintenance. Whether this is due to its editing activity or, most likely, to other ADAR1 functions[45] remains to be established. However, when the ADAR1-silenced U-937 cells were PMA-differentiated *in vitro*, ADAR1 expression failed to increase without this interfering with the course of cell differentiation, indicating a lack of ADAR1 involvement within this process. This finding is further confirmed by the lack of increase in ADAR1 expression, which remained stable in leukemia blasts undergoing *in vitro* PMA differentiation. On the contrary, ADAR2 also in this case was strongly upregulated, as already observed in the two myeloid cell lines. The above observation indicates that, differently from ADAR1, ADAR2 is not necessary or even detrimental for blasts homeostasis, as suggested by the observation that ADAR2 is eliminated through the ubiquitin–proteasome degradation pathway. However, ADAR2 might play some role afterwards when cells stop duplicating and proceed through differentiation.

As for the editing activity, most of the A-to-I editing sites in the literature were identified by computational analysis of sequence data without experimental validation.[46] By deep-sequencing analysis, we observed that there was a correlation between the expression of ADARs and the global editing in U-937 cells. When some targets, selected for being highly edited at the end of PMA treatment, were experimentally verified, we observed that the increase in the expression of ADAR1 in both U-937 and THP1 cell lines correlated with the editing activity on specific ADAR1 targets, such as AZIN1. This observation was corroborated by the strong correlation observed between AZIN1 editing and ADAR1 mRNA expression in blasts from AML patients. However, hampering of its upregulation and activity does not appear to impact myeloid cell differentiation.

The increment in ADAR2 expression along differentiation correlates with the global editing increase. We then searched for specific targets for ADAR2 in the recoding of the selected targets. Although we could not rely on ADAR2-silenced cells, nevertheless, using alternative approaches, we observed that, while AZIN1 and CCNI are specific targets for ADAR1, COG3 and RHOA, the latter at least at aa position 171, are edited by ADAR2. As a matter of fact, we report here that the activity of the two ADARs on these specific targets can be independent of each other. Of note, RHOA is highly edited, and it has been reported that another protein of the same pathway, RhoGTPase activating protein (ARHGAP26), is also regulated through ADAR editing, suggesting that this pathway can be a special target of the ADARs.[47] Interestingly, the edited form of AZIN1 has been reported to concur to proliferation and transformation in hepatocarcinoma cells.[48] In myeloid cells, the increase in AZIN1 editing mostly occurs in differentiated cells.

Most intriguing, ADAR2 is only detectable in cells at more mature stage of differentiation. It has been demonstrated that ADAR2 is posttranscriptionally regulated by the E3 ubiquitin ligase WWP2, whose action is counteracted by the phosphorylation-dependent prolyl-isomerase PIN1, a positive regulator required for the nuclear localization and stability of ADAR2.[34] In both U-937 and THP1 cells, proteasome inhibition revealed that the absence of ADAR2 in proliferating leukemia cell lines is due to its catabolism. We investigated whether PIN1 and WWP2 were differently expressed in U-937 undergoing PMA treatment and found that WWP2 mRNA was virtually absent (< 10 FPKM) at any time, whereas PIN1 was 10 times more abundant without significant variation during the time course. It is still possible, although unlikely, that the same degradation pathway is active in our system, or that another E3 ubiquitin ligase is responsible for the catabolism of ADAR2 in these cells. This aspect needs further investigation.

In summary, we show here for the first time that ADARs expression and activity are modulated during myeloid cell differentiation. This observation is also supported by the finding that peripheral blood monocytes show editing at selected recoding targets at level similar to that observed in *in vitro* differentiated leukemic cells. Furthermore, these observations candidate ADAR2 as a novel marker for myeloid blasts cell differentiation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Ministero della Università e Ricerca Scientifica (PRIN: 2012NA9E9Y_0029) by Associazione Italiana Ricerca sul Cancro (AIRC to AG) and by the Flagship InterOmics project. Publication costs for open access have been funded by the Flagship InterOmics project.

REFERENCES

1 Bass BL. RNA editing by adenosine deaminases that act on RNA. *Annu Rev Biochem* 2002; **71**: 817–846.
2 Wulff BE, Sakurai M, Nishikura K. Elucidating the inosinome: global approaches to adenosine-to-inosine RNA editing. *Nat Rev Genet* 2011; **12**: 81–85.
3 Nishikura K. Functions and regulation of RNA editing by ADAR deaminases. *Annu Rev Biochem* 2010; **79**: 321–349.

4 Patterson JB, Samuel CE. Expression and regulation by interferon of a double-stranded-RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. *Mol Cell Biol* 1995; **15**: 5376–5388.
5 Desterro JM, Keegan LP, Lafarga M, Berciano MT, O'Connell M, Carmo-Fonseca M. Dynamic association of RNA-editing enzymes with the nucleolus. *J Cell Sci* 2003; **116**: 1805–1818.
6 Athanasiadis A, Rich A, Maas S. Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome. *PLoS Biol* 2004; **2**: e391.
7 Dawson TR, Sansam CL, Emeson RB. Structure and sequence determinants required for the RNA editing of ADAR2 substrates. *J Biol Chem* 2004; **279**: 4941–4951.
8 Thomas JM, Beal PA. How do ADARs bind RNA? New protein-RNA structures illuminate substrate recognition by the RNA editing ADARs. *Bioessays* 2017; e-pub ahead of print 20 February 2017; doi: 10.1002/bies.201600187.
9 Higuchi M, Maas S, Single FN, Hartner J, Rozov A, Burnashev N *et al.* Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* 2000; **406**: 78–81.
10 Mannion N, Arieti F, Gallo A, Keegan LP, O'Connell MA. New insights into the biological role of mammalian ADARs; the RNA editing proteins. *Biomolecules* 2015; **5**: 2338–2362.
11 Tomaselli S, Galeano F, Massimi L, Di Rocco C, Lauriola L, Mastronuzzi A *et al.* ADAR2 editing activity in newly diagnosed versus relapsed pediatric high-grade astrocytomas. *BMC Cancer* 2013; **13**: 255.
12 Gallo A. RNA editing enters the limelight in cancer. *Nat Med* 2013; **19**: 130–131.
13 Crow YJ, Chase DS, Lowenstein Schmidt J, Szynkiewicz M, Forte GM, Gornall HL *et al.* Characterization of human disease phenotypes associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR, and IFIH1. *Am J Med Genet A* 2015; **167A**: 296–312.
14 Hwang T, Park CK, Leung AK, Gao Y, Hyde TM, Kleinman JE *et al.* Dynamic regulation of RNA editing in human brain development and disease. *Nat Neurosci* 2016; **19**: 1093–1099.
15 Zipeto MA, Jiang Q, Melese E, Jamieson CH. RNA rewriting, recoding, and rewiring in human disease. *Trends Mol Med* 2015; **21**: 549–559.
16 Slotkin W, Nishikura K, Adenosine-to-inosine RNA. Editing and human disease. *Genome Med* 2013; **5**: 105.
17 Wang Q, Khillan J, Gadue P, Nishikura K. Requirement of the RNA editing deaminase ADAR1 gene for embryonic erythropoiesis. *Science* 2000; **290**: 1765–1768.
18 Liddicoat BJ, Hartner JC, Piskol R, Ramaswami G, Chalk AM, Kingsley PD *et al.* Adenosine-to-inosine RNA editing by ADAR1 is essential for normal murine erythropoiesis. *Exp Hematol* 2016; **44**: 947–963.
19 Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One* 2010; **5**: 1–10.
20 Cenci C, Barzotti R, Galeano F, Corbelli S, Rota R, Massimi L *et al.* Down-regulation of RNA editing in pediatric astrocytomas: ADAR2 editing activity inhibits cell migration and proliferation. *J Biol Chem* 2008; **283**: 7251–7260.
21 Wu TD, Nacu S. Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinformatics* 2010; **26**: 873–881.
22 Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* 2009; **25**: 2078–2079.
23 Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 2010; **28**: 511–515.
24 Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 2013; **31**: 46–53.
25 D'Antonio M, D'Onorio De Meo P, Pallocca M, Picardi E, D'Erchia AM, Calogero RA *et al.* RAP: RNA-Seq analysis pipeline, a new cloud-based NGS web application. *BMC Genomics* 2015; **16**: S3.
26 Picardi E, Pesole G. REDItools: high-throughput RNA editing detection made easy. *Bioinformatics* 2013; **29**: 1813–1814.
27 Bazak L, Levanon EY, Eisenberg E. Genome-wide analysis of Alu editability. *Nucleic Acids Res* 2014; **42**: 6876–6884.
28 Picardi E, D'Erchia AM, Lo Giudice C, Pesole G. REDIportal: a comprehensive database of A-to-I RNA editing events in humans. *Nucleic Acids Res* 2016; **45**: D750–D757.
29 Chen L, Li Y, Lin CH, Chan TH, Chow RK, Song Y *et al.* Recoding RNA editing of AZIN1 predisposes to hepatocellular carcinoma. *Nat Med* 2013; **19**: 209–216.
30 Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U937). *Int J Cancer* 1976; **17**: 565–577.
31 Baek YS, Haas S, Hackstein H, Bein G, Hernandez-Santana M, Lehrach H *et al.* Identification of novel transcriptional regulators involved in macrophage differentiation and activation in U937 cells. *BMC Immunol* 2009; **10**: 18.
32 Camilli G, Cassotta A, Battella S, Palmieri G, Santoni A, Paladini F *et al.* Regulation and trafficking of the HLA-E molecules during monocyte-macrophage differentiation. *J Leukoc Biol* 2016; **99**: 121–130.
33 Steinman RA, Huang J, Yaroslavskiy B, Goff JP, Ball ED, Nguyen A. Regulation of p21(WAF1) expression during normal myeloid differentiation. *Blood* 1998; **91**: 4531–4542.
34 Marcucci R, Brindle J, Paro S, Casadio A, Hempel S, Morrice N *et al.* Pin1 and WWP2 regulate GluR2 Q/R site RNA editing by ADAR2 with opposing effects. *EMBO J* 2011; **30**: 4211–4222.
35 Paz-Yaacov N, Bazak L, Buchumenski I, Porath HT, Danan-Gotthold M, Knisbacher BA *et al.* Elevated RNA editing activity is a major contributor to transcriptomic diversity in tumors. *Cell Rep* 2015; **13**: 267–276.
36 Orlowski RJ, O'Rourke KS, Olorenshaw I, HawkinsGA, Maas S, Laxminarayana D. Altered editing in cyclic nucleotide phosphodiesterase 8A1 gene transcripts of systemic lupus erythematosus T lymphocytes. *Immunology* 2008; **125**: 408–419.
37 Laxminarayana D, O'Rourke KS, Maas S, Olorenshaw I. Altered editing in RNA editing adenosine deaminase ADAR2 gene transcripts of systemic lupus erythematosus T lymphocytes. *Immunology* 2007; **121**: 359–369.
38 Shifera AS, Hardin JA. PMA induces expression from the herpes simplex virus thymidine kinase promoter via the activation of JNK and ERK in the presence of adenoviral E1A proteins. *Arch Biochem Biophys* 2009; **490**: 145–157.
39 Vogler M. BCL2A1: the underdog in the BCL2 family. *Cell Death Differ* 2012; **19**: 67–74.
40 Tomaselli S, Galeano F, Alon S, Raho S, Galardi S, Polito VA *et al.* Modulation of microRNA editing, expression and processing by ADAR2 deaminase in glioblastoma. *Genome Biol* 2015; **16**: 5.
41 XuFeng R, Boyer MJ, Shen H, Li Y, Yu H, Gao Y *et al.* ADAR1 is required for hematopoietic progenitor cell survival via RNA editing. *PNAS* 2009; **106**: 17763–17768.
42 Jiang Q, Crews LA, Barrett CL, Chun HJ, Court AC, Isquith JM *et al.* ADAR1 promotes malignant progenitor reprogramming in chronic myeloid leukemia. *Proc Natl Acad Sci USA* 2013; **110**: 1041–1046.
43 Hartner JC, Walkley CR, Lu J, Orkin SH. ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. *Nat Immunol* 2009; **10**: 109–115.
44 Zipeto MA, Court AC, Sadarangani A, Delos Santos NP, Balaian L, Chun HJ *et al.* ADAR1 activation drives leukemia stem cell self-renewal by impairing let-7 biogenesis. *Cell Stem Cell* 2016; **19**: 177–191.
45 Bahn JH, Ahn J, Lin X, Zhang Q, Lee JH, Civelek M *et al.* Genomic analysis of ADAR1 binding and its involvement in multiple RNA processing pathways. *Nat Commun* 2015; **6**: 6355.
46 Pinto Y, Cohen HY, Levanon EY. Mammalian conserved ADAR targets comprise only a small fragment of the human editosome. *Genome Biol* 2014; **15**: R5.
47 Wang Q, Hui H, Guo Z, Zhang W, Hu Y, He T *et al.* ADAR1 regulates ARHGAP26 gene expression through RNA editing by disrupting miR-30b-3p and miR-573 binding. *RNA* 2013; **19**: 1525–1536.
48 Li Y, Chen L, Chan TH, Guan XY. Hepatocellular carcinoma: transcriptome diversity regulated by RNA editing. *Int J Biochem Cell Biol* 2013; **45**: 1843–1848.
49 Aberle H, Bauer A, Stappert J, Kispert A, Kemier R. β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* 1997; **16**: 3797–3804.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)